Tissue culture of *Solanum laciniatum*

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Abstract Methods for the in vitro propagation of complete *Solanum laciniatum* Ait. plants from callus, leaves, shoots, and apical meristem tips are described. Callus proliferation occurred on Murashige & Skoog medium (MS) supplemented with 10^{-6}M 6-benzylaminopurine (BAP) and 10^{-6}M β-naphthoxyacetic acid. Maximum shoot organogenesis from this callus occurred on MS + 10^{-5}M BAP. Adventitious shoot proliferation from both leaves and shoots was also possible on MS + 10^{-5}M BAP, as was the development of apical meristem tips (0.3–0.5 mm long) into shoots. Cultured shoots readily formed roots when subcultured on to MS medium without plant growth regulators and were successfully transferred to a greenhouse.

Keywords *Solanum laciniatum*; plant tissue culture; in vitro culture; micropropagation; callus growth; callus organogenesis; leaf culture; shoot culture; apical meristem-tip culture; auxin/cytokinin interaction

INTRODUCTION

Plant tissue culture techniques have been exploited in numerous ways in applied botany. In addition to the rapid clonal propagation of plants (Murashige 1974) they have important applications in crop improvement. Meristem-tip culture used in association with thermotherapy has become a standard technique for the propagation of disease-free plants from pathogen-infected stocks (Quak 1977), whereas in vitro procedures for the induction and/or selection of desirable traits in crop plants provide very useful tools to supplement conventional plant breeding methods (Scowcroft 1977). However, before any attempts can be made to fulfil such crop improvement objectives, it is necessary to develop reliable methods for the in vitro micropropagation of the particular species/cultivar. In addition to rapid clonal propagation involving shoot proliferation and subsequent rooting, procedures that should be developed include the regeneration of complete plants from meristem tips (for pathogen elimination), as well as callus growth and organogenesis (for the selection and subsequent regeneration of mutant cell lines).

This work was undertaken to develop in vitro culture methods for *Solanum laciniatum* Ait., a native shrub/small tree of New Zealand and southern Australia. The species is cultivated for the extraction of solasodine, a steroidal alkaloid used as a raw material for the synthesis of steroidal drugs (Mann 1978).

There have been several previous studies on the in vitro culture of *S. laciniatum*. Rerábek (1964, 1967) has reported the induction of callus from seedling roots, whereas others have maintained proliferating callus for studies on alkaloid production in such cultures (Vágújfalvi et al. 1971, Zenk 1978, Hosoda & Yatazawa 1979, Hosoda et al. 1979. Davies & Dale (1979) examined the combined action of several plant growth regulators on callus induction and adventitious organ development from leaf disc explants. Nevertheless, callus organogenesis, the regeneration of complete plants from meristem tips, and continuous shoot proliferation for rapid clonal micropropagation, all of which are pertinent to crop improvement, have not been reported previously.

MATERIALS AND METHODS

Plant material was obtained from *S. laciniatum* growing on the Lincoln College campus, Canterbury, New Zealand. All plant material was surface sterilised by immersion (with stirring) in 20% "Janola" (a commercial bleach containing 3% sodium hypochlorite) for 15 min, followed by 2 rinses in sterile distilled water. Cultures were initiated from nodal segments (1–2 cm long) with single axillary buds and 1 cm^2 leaf segments cut from fully expanded leaves. Cultures were established and subsequently subcultured under aseptic conditions in a sterile, laminar air-flow cabinet.

The basal culture medium consisted of the inorganic salts from the MS medium (Murashige & Skoog 1962), except that the ferric iron 200 × stock solution contained $FeSO_4 \cdot 7H_2O$ (5.56 g/L and $Na_2EDTA \cdot 2H_2O$ (7.45 g/L). To this, the following organic constituents were added: thiamine·HCl (0.1 mg/L), nicotinic acid (0.5 mg/L), pyridoxine·HCl (0.5 mg/L), glycine (2 mg/L), *myo*-inositol (100

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mg/L), and sucrose (3% (w/v)). After the work of Davies & Dale (1979) had been consulted and preliminary experiments had been carried out, the most effective cytokinin and auxin for inducing in vitro growth responses from *S. laciniatum* tissue were found to be BAP (6-benzylaminopurine) and NOA (β-naphthoxyacetic acid) respectively. These plant growth regulators were used in various combinations in concentrations ranging from 10^{-6}M to 5×10^{-5}M. The pH of the culture medium was adjusted to 5.6 with 1M NaOH and, when required, Davis (New Zealand) agar (1.0% (w/v)) was added. Gelled medium was dispensed (in 20-ml aliquots) into 100-ml Erlenmeyer flasks, and liquid medium (in 10-ml aliquots) into 2.5 × 15 cm test tubes with filter paper bridges (constructed with Whatman no. 1 filter paper). The mouths of all culture vessels were plugged with cotton wool, covered with aluminium foil caps, and then sterilised by autoclaving at 103 kPa for 15 min.

All cultures were incubated at 25°C under cool-white fluorescent tubes providing 48 $\mu E.m^{-2}.sec^{-1}$ at the culture level, with a 16 hr light : 8 hr dark photoperiod.

EXPERIMENTAL AND RESULTS

Culture establishment

The initial leaf and nodal segments were explanted into gelled MS medium plus various BAP concentrations. After 2–3 weeks, adventitious shoots and a little callus had developed from the explants. By 5 weeks, extensive shoot and/or callus formation had occurred, especially from the leaf segments. The axillary buds of the nodal segments showed only slow elongation, however, some adventitious shoots developed from the stem regions within the gelled medium. The extent of shoot proliferation increased with increasing BAP concentration from 10^{-6}M (primarily callus growth with a few shoots) through 5×10^{-6}M (moderate shoot development) to 10^{-5}M (maximum shoot proliferation). However, further increases in concentration to 5×10^{-5}M BAP inhibited all culture growth.

Callus growth and organogenesis

Small pieces of leaf callus, approx. 5 mm^3 (mean dry weight ± standard deviation = 0.0069 ± 0.0014 g, n = 10), were subcultured on to gelled MS medium with 5 concentrations (0, 10^{-6}M, 5×10^{-6}M, 10^{-5}M, 5×10^{-5}M) of BAP and NOA in all possible combinations and cultured for 5 weeks. The results are summarised in Fig. 1. Callus proliferation was greatest when the MS medium was supplemented with (10^{-6}M) NOA and (10^{-6}M) BAP. Increasing the auxin concentration decreased callus growth, whereas increasing the cytokinin concentration induced shoot differentiation. Shoot organogenesis primarily occurred when the concentration of cytokinin was equal to, or greater than, the auxin concentration. Except for the highest BAP concentration (5×10^{-5}M) which was inhibitory to all growth, shoot organogenesis increased with increasing cytokinin:organogenesis from callus occurred when the MS medium was supplemented with 10^{-5}M BAP and no, or low, auxin levels. Root organogenesis occurred on both the hormone-free medium and at the lowest auxin concentrations (10^{-6}M, 5×10^{-6}M) in the absence of cytokinin. Increasing the auxin concentration inhibited root organogenesis. It is probable that root organogenesis occurs more in response to the absence of cytokinins than the presence of auxins.

Shoot proliferation

Small clumps of several shoots, approx. 1 cm^3 (mean dry weight ± standard deviation = 0.024 ± 0.004 g, n = 10), were subcultured, pushed into gelled MS medium with a range of BAP and NOA concentrations in various combinations, and cultured for 5 weeks. The concentrations of plant growth regulators were chosen after the previous experiment and were those considered most likely to promote shoot proliferation. The results are summarised in Table 1. The extent of shoot proliferation increased with increases in BAP to 10^{-5}M, then declined. This trend was apparent with respect to both the number of shoots formed and the dry weight of cultures. Although the addition of auxin to this cytokinin concentration had little effect on culture dry weight, there was a substantial decrease in the number of shoots formed. This is indicative of a change from shoot proliferation to callus growth.

When the masses of shoots formed were subcultured into small clumps of several shoots (approx. 1 cm^3) and pushed into gelled MS medium with 10^{-5}M BAP, they continued to proliferate adventitious shoots and some axillary shoots with a 6–7 fold increase every 4–5 weeks (Fig. 2A). Although fasciated shoots developed occasionally, normal shoots grew out with extended culture.

Development from meristem tips

Apical meristem tips (0.3–0.5 mm long) were aseptically dissected from previously cultured shoots under a stereoscopic microscope. Attempts at culturing these tips directly on a gelled medium immediately after dissection were unsuccessful and resulted in their desiccation and senescence. However, when cultured on filter paper bridges with

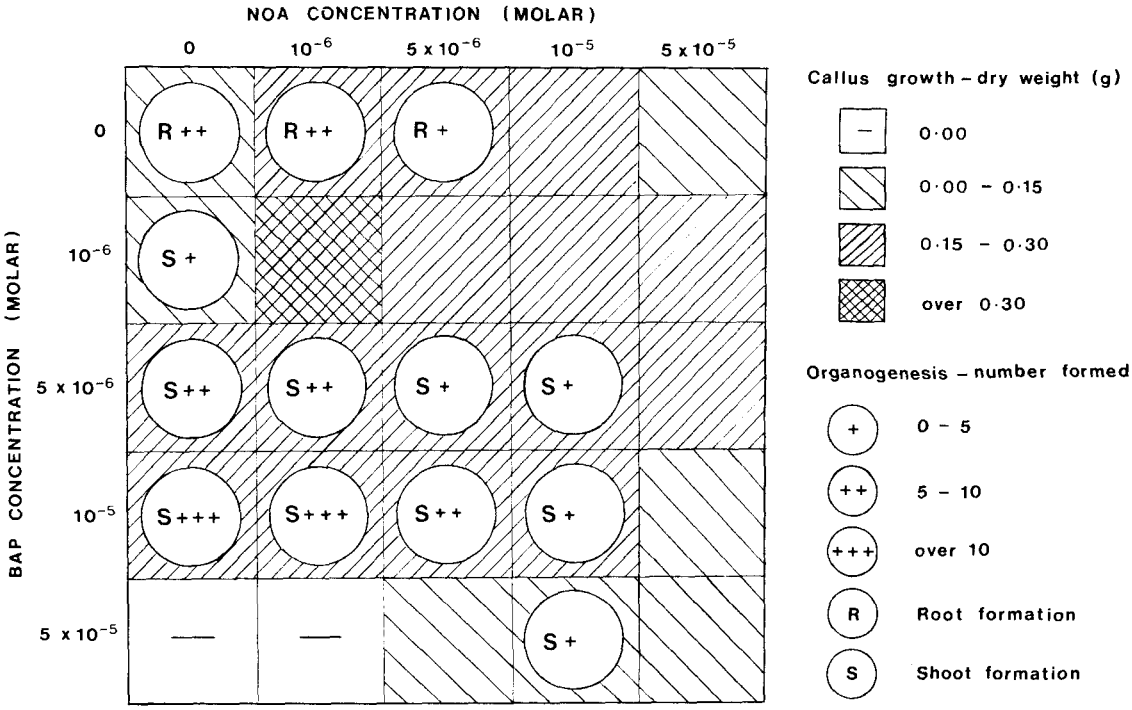

Fig. 1 Effects of BAP and NOA alone and in 16 different combinations on callus growth and organogenesis. All figures are the means of 5 replicates.

Table 1 Shoot proliferation after 5 weeks culture. Figures are means ± s.d. for 10 replicate cultures.

Medium	Number of shoots formed	Dry weight of cultures (g)
MS + 10^{-6}M BAP	6.5 ± 3.2	0.142 ± 0.059
MS + 5 × 10^{-6}M BAP	11.6 ± 2.7	0.297 ± 0.092
MS + 10^{-5}M BAP	18.3 ± 3.7	0.371 ± 0.074
MS + 5 × 10^{-5}M BAP	3.0 ± 1.6	0.123 ± 0.085
MS + 10^{-5}M BAP + 10^{-6}M NOA	15.5 ± 3.7	0.367 ± 0.088
MS + 10^{-5}M BAP + 5 × 10^{-6}M NOA	10.3 ± 2.1	0.429 ± 0.090

MS medium plus 10^{-5}M BAP, these meristem tips gradually grew to form small shoots after 1 month (Fig. 2B, C, D). At this stage they could be transferred on to a gelled medium (also MS plus 10^{-5}M BAP) where they further developed and produced several shoots. Altering the BAP concentration or including NOA in the medium reduced shoot development and/or induced callus formation.

Root formation on excised shoots

Single shoots, approx. 1 cm long, were excised from shoot proliferation cultures and transferred on to various gelled MS media modifications considered to have potential for inducing root formation. Root formation was evident after 10–12 days from the stem regions within the gelled media. The results after 3 weeks culture are summarised in Table 2.

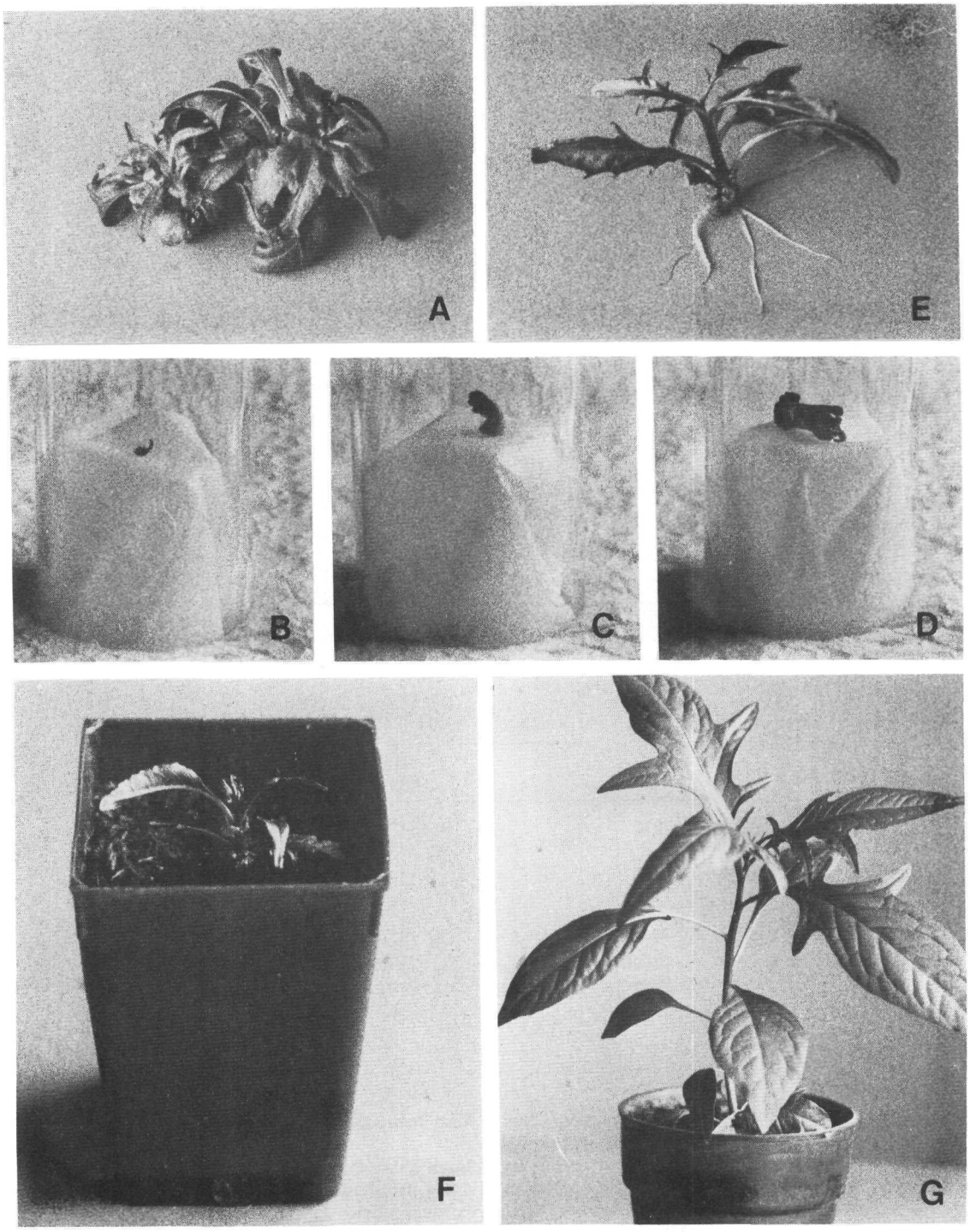

Fig. 2 (A):Shoot proliferation culture after 5 weeks on MS + 10^{-5}M BAP (× 2). (B–D): Development of apical meristem tips on MS + 10^{-5}M BAP (× 1.4), (B) after 2 weeks culture, (C) after 3 weeks culture, (D) after 4 weeks culture. (E): A rooted plantlet after 3 weeks on MS inorganic salts (× 2). (F): A plant after transfer to peat/sand mix (× 1). (G): A plant 2 months after transplantation from tissue culture (× 0.4).

Table 2 Root formation on excised shoots after 3 weeks culture, n = 10.

Medium	Number of shoots with roots	Mean number of roots per shoot ± s.d.
MS + 10^{-6}M NOA	4	1.1 ± 1.5
MS + 5 × 10^{-6}M NOA	5	1.0 ± 1.2
MS inorganic salts	9	2.3 ± 1.5
Half-strength MS	9	3.2 ± 1.7
MS	10	3.4 ± 1.6

Only some shoots developed roots when subcultured on to MS medium with low auxin concentrations. The formation of roots was considerably more successful on the MS medium without plant growth regulators, half-strength MS medium, or the inorganic salts only of the MS medium (Fig. 2E).

Transplantation from tissue culture

Plantlets were maintained on the rooting media for approx. 10–14 days after root initiation to allow further shoot and root elongation. The gelled medium was then carefully washed from around the roots and the plantlets transferred to a sterilised peat–sand (60:40) mix in 25 cm^2 × 7 cm-deep propagation tubes (Fig. 2F). To maintain a high humidity around the plantlets, the propagation tubes were enclosed in plastic bags after the mix was watered. After 7–10 days in the culture room, the plastic bags were gradually opened and finally removed a further 7–10 days later. One month after the plantlets had been transferred from tissue culture, shoot height had increased from approx. 2 cm to 5 cm and supported well developed roots. These plants were repotted and transferred to a greenhouse (Fig. 2G).

DISCUSSION

Although *S. laciniatum* is readily propagated by conventional techniques, the methods presented here for its rapid in vitro micropropagation are of value, especially if used in conjunction with pathogen elimination and breeding/selection programmes in which high-health or genetically superior plants are initially in low supply. Conservative estimates, using the procedures described here, indicate that over 25 million plantlets could potentially be produced from a single proliferating culture in 1 year. This system of micropropagation relies on the proliferation of adventitious shoots in addition to axillary bud development. There is often considered to be a higher probability of producing genetically variant plants (especially chimeras) through adventitious bud formation compared with proliferation of axillary shoots (D'Amato 1978). Nevertheless, the plants produced via adventitious shoots appear visually uniform in their leaf morphology and overall growth form. *S. laciniatum* plants propagated via adventitious shoots from in vitro leaf segments are known to maintain a stable karyotype (Davies & Dale 1979). Furthermore, severe "cutting back" of *S. laciniatum* plants, a feature necessary during their harvest for solasodine extraction (Davies & Mann 1978), often results in the development of adventitious shoots (unpubl. pers. observ.). Therefore, in vitro micropropagation via adventitious shoots is unlikely to cause major problems. However, if adventitious shoot formation in vitro is found to be undesirable, then the tendency for their development can be reduced by lowering the cytokinin concentration in the medium (from 10^{-5}M to 10^{-6}M BAP), although some callusing then occurs at the base of the explants. On this medium, shoots arising solely from axillary positions, which are not of adventitious or callus origin, can be easily selected for further subculturing, but the potential rate of propagation is greatly reduced.

The in vitro rooting of excised shoots was successful on MS media free of plant growth regulators. The ability of the shoots to root on the MS inorganic salts is especially interesting as this also allows the plantlets to fully establish their photosynthetic systems before transplantation. This therefore overcomes one of the difficulties associated with plantlet survival subsequent to transplantation from tissue culture (i.e., the switch from heterotrophic to photoautotrophic nutrition).

Rapid callus growth and organogenesis was readily achieved for *S. laciniatum*. The formation of shoots and roots was dependant on the relative concentration of auxin and cytokinin. Although this concept of auxin–cytokinin balance in the regulation of organogenesis has been confirmed many times (Gresshoff 1978), its demonstration in a woody species is of interest.

Callus cultures are renowned for chromosome number variation and consequently are considered least appropriate for clonal micropropagation (D'Amato 1978). However, it is in the context of regenerating complete plants from mutant cell lines selected in vitro that these procedures should prove useful. Several variant callus lines of *S. laciniatum* that have recently been isolated yield considerably higher levels of alkaloids than is usual in cell culture (Zenk 1978). The regeneration of plants from such

callus lines will assist in determining whether this new phenotype has resulted from an epigenetic or a genetic modification.

Several viruses are commonly found in *S. laciniatum* (Thomson 1976, 1979). The development of shoots from meristem tips in association with their subsequent rapid micropropagation into complete plants offers a potential technique for pathogen elimination.

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